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(57) Abstract

The present invention is based on the unexpected observation that DNA methylation, particularly at cytosine residues, that regulates gene activation states and the rate of development and differentiation of cells and organisms, can be altered by providing methioninase to a cell, tissue or organism. In addition, other methylation reactions within a cell can be altered by providing methioninase to a cell, tissue or organism. Based on these observations, the present invention provides methods of activating inactive genes, methods for decreasing the rate of development of cell, tissues or an organism, methods of treating obesity and methods of treating Parkinson's disease and other neurological disorders, by either providing a methioninase containing composition to a cell, tissue or organism or by introducing a methioninase encoding DNA molecule into the cell, tissue or organism. The present invention further provides organisms and cells that have been altered such that their rate of maturation is decreased and/or the degree of methylation of cellular constituents (DNA, RNA, lipids, proteins small cellular molecules, etc.) is decreased.

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METHODS FOR ACTIVATING INACTIVE GENES AND METHODS FOR ALTERING THE RATE OF DEVELOPMENT OF CELLS, TISSUES AND ORGANISMS

TECHNICAL FIELD

The present application is in the fields of gene activation/inactivation, cellular development and organism aging. The present invention particularly provides methods for activating inactive genes and methods for altering the rate at which cells, tissues and organisms differentiate, mature, develop and age based on the use of methioninase to decrease the amount of DNA methylation and the methylation of proteins and other cellular constituents present in a cell or organism.

BACKGROUND ART

Methionine And Aging

Aging has also been associated with changes in DNA methylation. Issa and Baylin (Issa, J-P. *et al. Nature Medicine* (1996) 2:281-282) have recently described progressive hypermethylation of the estrogen receptor gene in aging colon and have related it to cancer (Issa, J. *et al. Nature Genetics* (1994) 7:538) and to hematopoietic neoplasms (Issa, J. *et al. Cancer Res* (1996) 56:973-977). It is likely that other genes will be similarly affected by aging. The promoter methylation of the IGF2 gene itself is greatly increased in human colon as a function of aging (Issa, J-P. *et al. Nature Medicine* (1996) 2:281-282).

Orentreich *et al.* have reported (Orentreich, N. *et al. J Nutr* (1993) 123:269-274) that lifelong reduction of L-methionine, from 0.86 to 0.17% of the diet results in a 30% longer life span of male Fischer 344 rats. Methionine restriction eliminated weight gain, even though food intake was larger. Increasing the energy intake of rats fed 0.17% methionine failed to increase their rate of growth, whereas restricting the food intake of 0.85% methionine-fed rats to that of 0.17% methionine-fed animals did not reduce growth, indicating that food restriction was not a factor in life span extension in these experiments (Orentreich, N. *et al. J Nutr* (1993) 123:269-274).

Methionine And Obesity

The work of Orentreich (Orentreich, N. *et al. J Nutr* (1993) 123:269-274) also suggests that methionine levels can be a factor contributing to obesity, since methionine deprivation controlled weight gain so exquisitely in the rats.

Recent work has indicated the importance of hormone control of obesity including the hormones leptin, neuropeptide Y (NPY) and a specific melanocortin receptor as key components of the systems in the brain that regulate body weight (Friedman, J.M. *Nature* (1997) 385:119-120).

Fan *et al.* (Fan, W. *et al. Nature* (1997) 385:165-168) and Huszar *et al.* (Huszar, D. *et al. Cell* (1997) 88:131-141) have shown that the melanocortin-4 receptor and its peptide ligand, melanocyte-stimulating hormone (MSH) are important in the pathogenesis of obesity in mice with the mutation yellow agouti (Friedman, J.M. *Nature* (1997) 385:119-120). Erickson *et al.* (Erickson, J.C. *et al. Science* (1996) 274:1704-1707) have reported NPT can reduce obesity and other abnormalities that are seen in mice mutated in the *obese* (*ob*) gene.

The *agouti* gene's product is a hair-follicle secreted factor that is overexpressed in these mice (Bultman, S.J. *et al. Cell* (1992) 71:1195-1204). When it is overexpressed in the hair follicles, the agouti peptide inhibits the effects of melanocyte stimulating hormone (MSH) on melanocortin-1 receptors that leads to the yellow coat color (Lu, D. *et al. Nature* (1994) 371:799-802). The agouti peptide also inhibits the action of MSH on the MC-4 receptor in the brain (Lu, D. *et al. Nature* (1994) 371:799-802; Mountjoy K.G. *et al. Mol Endocrinol* (1994) 8:1298-1308). Mice with mutations in the MC-4 receptor are as obese as the agouti mice, but they do not have yellow coats (Huszar, D. *et al. Cell* (1997) 88:131-141). Fan *et al.* (Fan, W. *et al. Nature* (1997) 385:165-168) have showed that MSH agonists inhibit food intake in normal and obese animals whereas MSH antagonists have the opposite effect. In addition, both agouti and *MC-4*-knockout mice have very high blood levels of another fat regulator -- leptin. So it seems that in the absence of a functional MC-4 receptor, animals no longer decrease their weight during increased concentrations of leptin in the blood (Issa, J-P. *et al. Nature Medicine* (1996) 2:281-282). It will be very

interesting to note how methionine starvation/rMETase-mediated block of weight gain interacts with those hormone regulators of obesity.

Methionine And Parkinson's Disease

S-adenosyl-L-methionine has been shown to cause Parkinson's disease-like effects that include hypokinesia, tremor, rigidity, and abnormal posture in rats when injected into the lateral ventricle (Crowell, Jr., B.G. *et al. Behavioral and neural biology* (1993) 59:186-193). S-adenosyl-L-methionine is the rate-limiting endogenous methyl donor for the methylation of dopamine (Crowell, Jr., B.G. *et al. Behavioral and neural biology* (1993) 59:186-193). Therefore S-adenosyl-L-methionine and methionine, which is its precursor, may play a role in Parkinson's disease (Crowell, Jr., B.G. *et al. Behavioral and neural biology* (1993) 59:186-193). A dose of 200 mg/kg L-dopa, the main therapeutic agent for Parkinson's disease, blocked the hypokinetic effects of S-adenosyl-L-methionine, but D-dopa, the inactive analog, showed no effect (Crowell, Jr., B.G. *et al. Behavioral and neural biology* (1993) 59:186-193). Therefore, these findings suggest that S-adenosyl-L-methionine-induced hypokinesia, and its associated symptomatology, may serve as a model for the study of Parkinsonism and it may in fact be involved in the etiology of the disease (Crowell, Jr., B.G. *et al. Behavioral and neural biology* (1993) 59:186-193).

S-adenosyl-L-methionine is the methyl donor used by catechol-*O*-methyltransferase (COMT) in the *O*-methylation of levodopa, dopamine (DA), and 3,4-dihydroxyphenylacetic acid (DOPAC) (Da Prada, M. *et al. Clinical Neuropharmacology* (1994) 17:S26-S37). Blockage of *O*-methylation of levodopa and DA can increase the bioavailability of levodopa and DA (Da Prada, M. *et al. Clinical Neuropharmacology* (1994) 17:S26-S37). This should improve the beneficial effect of levodopa for the therapy of Parkinson's disease (PD) (Da Prada, M. *et al. Clinical Neuropharmacology* (1994) 17:S26-S37). Methioninase could be used to reduce circulating methioninase levels such that COMT is optimally inhibited for this therapeutic effect.

Allain *et al.* (Allain, P. *et al. Toxicology* (1995) 16:527-530) compared the levels of cysteine and homocysteine in the plasma of healthy subjects and of patients

with Parkinson's disease treated by L-dopa and dopa decarboxylase inhibitors. The levels of cysteine in the plasma of controls and patients with Parkinson's disease were not statistically different but the level of homocysteine was higher in patients.

Methioninase (L-methionine-alpha-deamino-gamma mercaptomethane-lyase or methioninase) has been used to deplete serum methionine as a means for treating cancer (US Serial Nos. 08/424,300, 08/486,519 and 08/642,521). Many tumors have an absolute requirement for methionine. Depletion of methionine in tumor cells induces stasis and apoptosis of the tumor cell. Further, methioninase has been shown to be an effective tumor selective modulator of other chemotherapeutic agents.

In addition, methioninase has been used to reduce the potential for heart disease by lowering serum homocysteine levels. Chronic administration of methioninase alone or in combination with a methionine reduced diet, is effective at lowering the risk of heart disease, particularly in patients who normally have elevated homocysteine levels.

Despite the use of methionine to treat these two conditions, the effect of methioninase treatment on other cellular functions that use methionine has not been explored. In the present invention, methioninase is used to deplete methionine as a means for reducing its availability to cellular enzymes that use methionine. One example is the use of methionine depletion methods to reduce the amount of methylated DNA present in a cell, tissue or organism.

It is therefore the focus of the present invention to provide methods for decreasing the amount of DNA methylation, and the amount of methylation of other cellular constituents present in a cell, tissue or organism as means for 1) activating genes inactivated due to hypermethylation and/or 2) altering the rate at which cells, tissues and organisms differentiate, develop and age.

In other examples, methioninase is used to deplete serum methionine levels as a means for treating obesity and as a means for treating Parkinson's disease. Both of these conditions are mediated in part by enzymes that use methionine.

DISCLOSURE OF THE INVENTION

The present invention is based on the observations that DNA methylation plays important roles in 1) gene activation/inactivation, 2) the control of the rate of differentiation and development of cells, tissues and organisms and 3) regulating the aging process and other degenerative disorders. The invention is further based on the observation that methioninase can be used to decrease the amount of methylated DNA, and other methylated cellular constituents, present in a cell, tissue or organism. Based on these observations, the present invention provides methods of altering the amount of DNA methylation in a cell, tissue or organism as a means for activating inactive genes and/or altering the rate of maturation or aging of a cell.

In one method, methioninase is used to activate dormant or inactive genes as a means for restoring gene function. This application of the method of the present invention can be used to treat disease states caused by abnormal gene inactivation, to cure deficiencies by activating fetal genes and for increasing the topipotency of cells used in creating chimeric, transgenic and clonal animals.

In another method, methioninase is used to decrease the rate of differentiation, maturation and aging of cells, tissues or an organism. This application of the method of the present invention can be used to prolong the viability of tissues and organs used in transplants, in treating aging disorders modulated by increased gene inactivation, and in increasing the longevity of organisms such as mammals and plants.

In another method, methioninase is used to decrease the availability of S-adensyl-L-methionine to enzymes responsible for mediating Parkinson's disease. This application of the method of the present invention can be used reduce the rate of onset of Parkinson's disease, and to increase the effectiveness of agents used to treat Parkinson's disease.

In another method, methioninase is used to decrease the availability of methionine as a means for treating obesity.

In general, these methods involve either contacting the cells, tissues or organism with a methioninase composition or by introducing a methioninase encoding nucleic acid molecule into the cell, tissue or organism.

DESCRIPTION OF THE INVENTION

I. General Description

The present invention is based on the combined observations that: 1)DNA methylation, particularly at cytosine nucleotides, is involved in the activation/inactivation of the expression of genes within a cell and in regulating the rate of cellular and organism differentiation, development and maturation, particularly the rate at which organisms and cells age and mature; and 2) that methioninase can be used to decrease the amount of methylated DNA in a cell, tissue or organism. Based on these observations, the present invention provides methods for: 1) activating inactive genes and 2) decreasing the rate of differentiation, maturation and aging of a cell, tissue or organism. The invention is further based on the observations of the roles that serum methionine plays in mediating the effects of Parkinson's disease and obesity.

In general, the methods of the present invention used to alter the amount of methylated DNA, and other methylated cellular constituents, present in a cell, tissue or organism, comprise the step of providing methioninase to the cell, tissue or organism in an amount sufficient to decrease the amount of methylated DNA, and other methylated cellular constituents, present in the cell, tissue of organism. In one embodiment, a methioninase composition is used to decrease the amount of methionine available for the methylation reaction of DNA and the methylation reaction of other cellular constituents. In another embodiment, genetic engineering methods are used to introduce a nucleic acid molecule that encodes methioninase into the cells that are to be altered. The invention as set forth below will first describe various methods that can be used to alter the methylated DNA content, and other methylated constituents, in a cell, tissue or organism. The invention will then set forth a non-limiting description of situations in which it is desirable to alter the amount of methylated DNA, and other methylated cellular constituents, present in a cell, tissue or organism.

II. Methionine Depletion Method

The present invention is based on the ability to reduce serum methionine levels using either a methioninase containing composition or an expression unit that encodes methioninase. Normally, humans have a serum methionine level of 30 to 100uM. Using either of the methods disclosed herein, serum methionine levels can be reduced to concentration below about 10uM or less. In tumor treatment, serum methionine levels have been able to be reduce to almost undetectable levels (US Serial Nos. 08/424,300, 08/486,519 and 08/642,521). However, this degree of reduction is not needed for the present methods. Only a reduction to levels of below 30uM, preferably below about 20uM, most preferably to levels about 10uM is needed.

A. Methioninase Compositions

The amount of methylated DNA, and other methylated cellular constituents, present in a cell can be reduced by reducing the amount of methionine that is present in a cell, tissue or organism, or surrounding fluids, using a composition containing methioninase. Such methods rely on contacting a cell, tissue or organism, or the fluid surrounding a cell, tissue or organism, with a methioninase composition. Whereas prior methods used methioninase compositions as a means for treating cancer cells, the present invention uses art known and suggested methioninase compositions and delivery methods as a means of reducing the amount of DNA methylation, and other methylated cellular constituents, present in a cell, tissue or organism.

A therapeutic composition comprising a therapeutically effective amount of methioninase is used to reduce the amount of methionine present in a cell, tissue or organism to an amount sufficient to reduce the amount of methylated DNA, and/or other methylated cellular constituents, present. L-methioninase (L-methionine-alpha-deamino-gammamercaptomethane-lyase or methioninase) is an enzyme that degrades methionine by deamination and dethiomethylation. Methioninase activity can be measured at least by measuring the amount of alpha-ketobutyrate formed upon cleavage of methionine. One unit (U) of methioninase is defined as an amount of enzyme that produces 1 micromole of alpha-ketobutyrate per minute from methionine under the standard assay conditions described by Ito et al., J. Biochem., 79:1263-1272, 1976; and Soda, Analyt. Biochem. 25:228-235, 1968.

The methioninase used in the present methods can be prepared from a variety of sources, including being isolated directly from bacteria cultures, or being expressed from a recombinant DNA molecule encoding a methioninase protein. Bacterial sources of methioninase include *Pseudomonas putida*, *Pseudomonas ovalis*, and *Aeromonas sp.* and any other potential sources of methioninase. *P. putida* strains are commercially available from the ATCC and have accession numbers ATCC 8209 and ATCC 7955, respectively. The other bacteria are generally available from the academic research community. Purification of methioninase has been achieved by a variety of methods. See, for example, US Serial Nos. 08/424,300, 08/486,519 and 08/642,521, Kreis et al., Cancer Res., 33:1862-1865, 1973; Tanaka et al., FEBS Letters 66:307-311, 1976; Ito et al., J. Biochem. 79:1263-1272, 1976; Nakayama et al., Agric. Biol. Chem. 48:2367-2369, 1984; and Soda, Analyt. Biochem. 25:228-235, 1968. Particularly preferred is methioninase prepared from *P. putida*, or using an expression vector containing a DNA molecule isolated from *P. putida* that encodes methioninase. *P. putida* methioninase exhibits an apparent molecular weight when analyzed on PAGE-SDS under denaturing conditions of about 43 kilodaltons.

A preferred methioninase used in the present methods has a specific activity of about 10 to about 50 units (U) per mg protein and is substantially free of contaminating proteins and endotoxin, such as bacterial lipopolysaccharides. (For example see Tan *et al., Protein Exp. Purif.* 9:233-245 (1997)). By substantially free is meant less than about 10 nanograms (ng) endotoxin per milligram (mg) methioninase protein, preferably less than 1 ng endotoxin per mg methioninase, and more preferably less than 0.1 ng endotoxin per mg methioninase. Assays for determining the amount of endotoxin present in a sample are well known in the art.

In general, the embodiments that employ a therapeutic composition will use a composition that comprises a pharmaceutically/physiologically tolerable carrier together with substantially isolated methioninase, dissolved or dispersed therein as an active ingredient. In a preferred embodiment, the methioninase is formulated for sustained or continual release, such as in a transdermal or oral delivery system. In another form, methioninase can be conjugated to a polymer such as polyethylene glycol, prior to incorporation into a composition.

As used herein, the terms "pharmaceutically acceptable", "physiologically tolerable" and grammatical variations thereof, as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to or upon a mammal or human without the production of undesirable physiological effects such as nausea, dizziness, gastric upset, allergic reactions and the like.

Methods for preparing a pharmacological composition that contains an active ingredient dissolved or dispersed therein are known in the art and can readily be used to generate methioninase containing compositions. Typically such compositions are prepared as sterile injectables either as liquid solutions or suspensions, aqueous or non-aqueous, however, solid forms suitable for solution, or suspensions, in liquid prior to use can also be prepared. The preparation can also be emulsified. In addition, a therapeutic amount of methioninase can be present in an ointment or on a diffusible patch, such as a bandage, as to afford systemic, transdermal delivery of the methioninase.

The methioninase can be mixed with excipients that are pharmaceutically acceptable and compatible with the active ingredient and in amounts suitable for use in the therapeutic methods described herein. Suitable excipients are, for example, water, saline, dextrose, glycerol, or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like that enhance the effectiveness of the active ingredient.

The methioninase used can be in the form of a pharmaceutically acceptable salt or a polymer conjugated form, such as in the use of pegylated methioninase (for example see US Serial Nos. 08/424,300, 08/486,519 and 08/642,521). Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide) that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, tartaric, mandelic and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine and the like.

Physiologically tolerable carriers are well known in the art. Exemplary of liquid carriers are sterile aqueous solutions that contain no materials in addition to the active ingredients and water, or contain a buffer such as sodium phosphate at physiological pH value, physiological saline or both, such as phosphate-buffered saline. Still further, aqueous carriers can contain more than one buffer salt, as well as salts such as sodium and potassium chlorides, dextrose, propylene glycol, polyethylene glycol and other solutes.

Liquid compositions can also contain liquid phases in addition to and to the exclusion of water, as described herein. Exemplary of such additional liquid phases are glycerin, vegetable oils such as cottonseed oil, organic esters such as ethyl oleate, and water-oil emulsions, particularly the liposome compositions described earlier.

Insofar as a methioninase composition can be used *in vivo* intravascularly, it is contemplated in one embodiment to formulate a therapeutic composition for controlled delivery of the methioninase, and optionally to shield the methioninase protein from degradation and other phenomenon which would reduce the serum half-life of therapeutically administered methioninase, such as that achieved by using therapeutic compositions containing a delivery vehicle such as polymers, polymeric vehicles, particulates, latexes, coacervates, ion-exchange resins, liposomes, enteric coatings, mediators, bioadhesives, microcapsules, hydrogels, and the like vehicles. Exemplary drug delivery vehicles including liposomes are described at least by Tarcha in "Polymers For Controlled Drug Delivery", CRC Press, Boca Raton, 1990.

Methionine depletion to reduce the amount of methylated DNA present in a cell can be conducted *in vivo*, in the circulation of a mammal, *in vitro* in cases where methionine depletion in tissue culture or other biological mediums is desired, and in ex vivo procedures where biological fluids, cells or tissues are manipulated outside the body and can then be subsequently returned to the body of the patient or mammal. The choice of how the methioninase composition is administered will be based primarily on the type of cell, tissue or organism that is being treated.

Because DNA methylation is dependent on methionine concentration, the depletion may be directed to the nutrient source for the cells, tissues or organism and not necessarily to the cells themselves. Therefore, in an *in vivo* application of the present

invention, the methioninase can be contacted with a biological fluid, such as the blood, lymphatic fluid, spinal fluid and the like bodily fluid where methionine depletion is desired.

Depletion of methionine from circulation, culture media, biological fluids or cells is conducted to reduce the amount of methionine accessible to the material being treated, and therefore comprises contacting the material to be depleted with a methionine-depleting amount of a methioninase composition under methionine-depleting conditions so as to degrade the ambient methionine in the material being contacted.

A methionine-depleting amount of a methioninase composition can vary widely depending upon the application, and typically depends upon the amount of methionine present in the cell, tissue, organism, or fluid, the desired rate of depletion, the tolerance of the material for exposure to methioninase and the desired effect that is to be achieved. Methionine levels in a material, the rate of methionine depletion from the material, and the amount of methylated DNA can readily be monitored by a variety of chemical and biochemical methods well known in the art. Exemplary methionine-depleting amounts can range from about 0.001 to about 100 units (U) of methioninase, preferably about 0.01 to about 10 U, and more preferably about 0.1 to about 5 U methioninase per milliliter (ml) of material to be treated.

The time period required to reduce the amount of methylation present depends on several factors, such as the amount of methioninase contacted with the cells or the medium containing the cells, the amount of methionine, specific activity of the enzyme, temperature and other reaction conditions affecting reaction rate, and the like parameters readily controllable by the practitioner. Typical time periods of about 10 minutes to about 30 days, preferably about 1 hour to 20 days, and more preferably about 1 to 10 days are used to treat cells or tissues. Longer periods, even systemic lifetime delivery, may be needed for treating an organism. In one use, methioninase is administered after food intake, at a period when serum methionine levels would normally increase.

The methioninase can be administered parenterally by injection or by gradual infusion over time. Methioninase can be administered intravenously, intraarterially, intraperitoneally, orally, intramuscularly, subcutaneously, intracavity, transdermally,

dermally, can be delivered by peristaltic means, can be administered by a pump connected to a catheter that may contain a potential biosensor or methionine, or by a controlled or sustained delivery system.

The therapeutic compositions containing methioninase are conventionally administered intravenously, as by injection of a unit dose, for example. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for the subject, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; i.e., carrier, or vehicle.

The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered depends on the subject to be treated, capacity of the subject's system to utilize the active ingredient, and degree of therapeutic effect desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosage ranges for systemic application are disclosed herein and depend on the route of administration. Suitable regimes for initial administration and booster shots are also contemplated and are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration. Exemplary multiple administrations are described herein and are particularly preferred to maintain continuously high serum and tissue levels of methioninase and conversely low serum and tissue levels of methionine. Alternatively, continuous intravenous infusion sufficient to maintain concentrations in the blood in the ranges specified for *in vivo* therapies are contemplated.

B. Synergistic Effect Of Multiple Methionine Depletion Methods

The synergy that occurs when two or more different methods for methionine depletion are used can be employed in the present method. Specifically, methioninase can be used alone or in combination with one or more additional methods that have been used to lower methionine levels. For example, methioninase can be used in combination with: (1) methionine starvation using methionine-free medium (*in vitro*) or

diet (*in vivo*), (2) competitive inhibitors of methionine to reduce the effective concentration of endogenous methionine, and (3) use of methionine precursors, such as homocysteine described herein, to increase the selectivity of any of the other three methionine depletion methods. Each of these other methods for reducing methioninase levels is disclosed in US Serial Nos. 08/424,300, 08/486,519 and 08/642,521.

Particularly preferred for maximum depletion of methionine and its resultant metabolites is the use of a methionine-free or reduced diet, further containing methionine precursors, plus the use of a competitive inhibitor of methionine utilizing enzymes.

For example, a reduced methionine diet may be used. Methionine-free media and methionine reduce/free diets are well known in the arts and can readily be used in combination with methioninase. An exemplary medium is Eagle's minimal essential medium (lacking methionine and choline chloride) with nonessential amino acids, such as is available from GIBCO. Methionine deficient amino acid diet foods are also commercially available, including diet TD 92077 available from Teklad, Inc.

When using a methionine-free/reduced nutrient (media or diet) in combination with methioninase, it may be desirable to use a methionine precursor such as homocysteine, or an analog thereof, to provide necessary nutritional supplements. Homocysteine can be added to nutrient medium at a concentration of about 5 to about 200 micromolar (uM), preferably about 10 to about 100 uM. Preferred methionine precursors useful in this embodiment include L-homocysteine-thiolactone, homocysteine, and 4-methylthio-2-oxobutanoic acid.

In addition to a methionine free/reduced diet, competitive inhibitors of methionine-utilizing enzymes are useful to synergistically may also be used to reduce the amount of methylated DNA in a cell. Methionine-utilizing enzymes useful for directing competitive inhibitors include S-adenosyl methionine decarboxylase, methionine t-RNA synthase and methionine adenosyltransferase.

Competitive inhibitors of methionine compete with methionine as a substrate for methionine-utilizing enzymes, and therefor act to inhibit any normal metabolic effect that endogenous methionine might produce by direct competition, i.e., inhibits methionine metabolism. Where the cell requires methionine and the attendant

metabolism of methionine, the competitive inhibitor acts to reduce the metabolism of methionine, necessitating higher methionine concentrations to yield the same effect observed where no inhibitor is present.

A competitive inhibitor of methionine can be any methionine derivative that functions as a classic competitive inhibitor. Typical competitive inhibitors include alkyl derivatives of methionine (i.e., alkylthionines), as where the methyl group of methionine is replaced with an ethyl group (ethionine), a propyl group (propthionine), a butyl group (buthionine), or a pentyl group (penthionine).

Also contemplated as useful methionine competitive inhibitors are cycloleucine and halogenated methionines. A typical halogenated methionine is selected from the group consisting of fluoromethionine, chloromethionine, bromomethionine and iodomethionine. Thus a contemplated competitive inhibitor of methionine is selected from the group consisting of alkylthionine, cycloleucine and a halogenated methionine, wherein said alkylthionine is not methionine.

An amount of a competitive inhibitor of methionine effective to competitively inhibit methionine is an amount to produce a reduction in the effective concentration of methionine. This amount is typically a molar excess relative to the methionine present, as shown in the Examples. Typically, this amount of inhibitor is in the range of a 10 to 1000 fold molar excess relative to the methionine concentration in the medium where methionine is to be competed, preferably at least a 20 fold molar excess, and more preferably at least a 50 fold molar excess. Where the inhibitor is to be used *in vivo*, the dosage is typically about 5 - 30 mg per kg of animal body weight, preferably about 25 mg/kg, as shown herein.

The amount of inhibitor required for effectiveness may vary depending upon the amount of endogenous methionine present at the time the inhibitor is administered. Typical amounts of inhibitor are from about 10 uM to about 1 mM.

C. Expression Of A Methioninase Encoding DNA Molecule

In addition to the use of a methioninase containing composition, methioninase can also be provided to a cell, tissue or organism by introducing into the cell, tissue or organism a DNA molecule that encodes methioninase. Methioninase encoding DNA

molecules have been described (US Serial Nos. 08/424,300, 08/486,519 and 08/642,521) and can readily be used in generating recombinant cells, tissues or organisms that express methioninase.

In general, altering a cell to express an introduced methioninase encoding DNA molecule rely on the use of molecular techniques. As used herein, molecular techniques exclude classical genetic techniques such as breeding/selection, identification of random mutagenesis and chemical mutagenesis techniques. Molecular techniques refer to procedures in which DNA is manipulated in a test tube during at least one stage of the process, such as the direct manipulation of DNA or the use of shuttle host such as bacterium. Such methods are well known in the art and are described in, for example, Sambrook, *et al. Molecular Cloning: a Laboratory Manual*, Cold Spring Harbor Press (1989). Some of the techniques that are used to alter a cell, tissue or organism are discussed in more detail below.

As provided above, one method that can be used to supply methioninase to a cell, tissue or organism employs expression units (or expression vectors or systems) to express an exogenously supplied methioninase gene in the cell, tissue or organism. Methods for generating expression units/systems/vectors are well known in the art and can readily be adapted for use in expressing a methioninase encoding sequence such that the amount of methylated DNA present in a cell, tissue or organism is reduced. Typically, such expression units employ a methioninase coding region, such as the methioninase gene from *Pseudomonas putida*, and one or more expression control elements. The choice of the control elements employed will be based on the desired location of expression, the amount/degree of expression desired, whether the expression is to be controlled (inducible control elements) and the cell, tissue or organism that is to be altered. A skilled artisan can readily use any appropriate vector/expression system in the present methods following art known methods.

The expression control elements used to regulate the expression of the methioninase coding region can either be the expression control element that is normally found associated with the methioninase gene or can be a heterologous expression control element. A variety of heterologous expression control elements are known in the art and can readily be used to make expression units for use in the

present invention. Such expression control elements include, but are not limited to, constitutive promoters, cell/tissue/organ-specific promoters, inducible promoters and secretion signal sequences. The expression unit may be further optimized by employing supplemental elements such as transcription terminators and/or enhancer elements.

Thus, for expression in cell, the expression units will typically contain, in addition to the methioninase encoding sequence, a promoter sequence, a transcription initiation site and a transcription termination sequence. Unique restriction enzyme sites at the 5' and 3' ends of the expression unit are typically included to allow for easy insertion into a preexisting vector.

The resulting expression unit is ligated into or otherwise constructed to be included in a vector which is appropriate for transforming the intended target cell, tissue or organism. The vector will also typically contain a selectable marker gene by which transformed cells can be identified. After transforming the target cell, tissue or organism cells, those cells having the vector will be identified by their ability to grow on a medium containing the particular antibiotic. Replication sequences, of bacterial or viral origin, are generally also included to allow the vector to be cloned in a bacterial or phage host, preferably a broad host range prokaryotic origin of replication is included. A selectable marker for bacteria can also be included to allow selection of bacterial cells bearing the desired construct. Suitable prokaryotic selectable markers also include resistance to antibiotics such as kanamycin or tetracycline.

A variety of methods are known in the art for introducing an expression unit into a cell, tissue or organism. The choice of the method employed will be based primarily on the types of cells, tissues or organism that it to be altered, the vector/expression system employed, and the conditions under which the expression unit is introduced into a cell, tissue or organism.

Methods for introducing an expression unit into a cell, tissue or organism include, but are not limited to, direct micro-injection into a cells using micropipettes (Crossway, Mol Gen Genetics (1985) **202**:179-185), agent complexed DNA uptake, such as in the use of polyethylene glycol or CaCl (Krens, *et al., Nature* (1982) **296**:72-74), high velocity ballistic penetration by small particles with the nucleic acid

either within the matrix of small beads or particles, or on the surface, is used (Klein, *et al., Nature* (1987) **327**:70-73), fusion with liposomes or other cells, such as bacterial spheroplasts, containing the expression unit, (Fraley, *et al., Proc Natl Acad Sci USA* (1982) **79**:1859-1863), and electroporation (From *et al., Proc Natl Acad Sci USA* (1985) **82**:5824). Other specific transformation systems have been developed for particular target host, such as the Agrobacterium transformation system employed with plants, plant cells and plant tissues.

Introduction of methioninase encoding expression unit into a cell results in a cell that expresses methioninase. Such cells can be used to deliver methioninase to a tissue or organism or can themselves be used, as described below, for a variety of purposes.

III. Use Of Methods For Reducing DNA methylation

There are many situations in which it is desirable to reduce the amount of methylated DNA present in a cell, tissue or organism. In general, such uses rely on the observation that increases in methylated DNA leads to gene inactivation and is responsible for mediating the processes of differentiation, maturation and aging. Accordingly, reducing the level of DNA methylation within a cell, tissue or organism can be used as a means for activating inactive genes, increasing the longevity of cells, tissues or an organism and decrease the rate of differentiation, maturation and development.

As used herein, a cell, tissue or organism is said to have a reduced or decreased amount of methylated DNA when the cell, tissue or organism has less methylated DNA than the non-altered cell, tissue or organism. Of the four nucleotides, cytosine has been seen to be methylated in cell, tissue or organisms. The present methods are therefore accomplished by targeting the methylation of cytosine.

What is contemplated is a reduction sufficient and effective to 1) increase the level of gene expression, or 2) decrease the rate of differentiation, maturation and aging in a manner useful for the purposes outlined herein. Thus any decrease in the amount of DNA methylation, so long as it results in an altered rate of gene expression, cell differentiation, development or maturation, or the aging process, is contemplated

by the present invention.

In the preferred embodiment, the method of the present invention will result in cells, tissues or an organism having from about 10 % to about 25 % reduction, more preferable from about 25 % to about 50 % reduction, most preferably from about 50 % to about 70 % or greater reduction in the amount of methylated DNA present when compared to non-treated cells, tissues or organism. A skilled artisan can readily use the general outline provided herein and known formulation/delivery methods to obtain such reductions in DNA methylation.

A. Gene Activation

There are many situations in which it is desirable to activate inactive genes. For example, many genes are only expressed in embryonic/fetal tissues. For example, a form of hemoglobin is expressed only in embryonic and fetal tissues. Such genes become inactive at later stages of development. Using the methods herein described, inactive genes can be activated as a means of treating a variety of pathological conditions.

Other pathological conditions have been show to be the result of gene inactivation. Each disease state that is currently known, or later shown to be mediated by gene inactivation can be treated by activating the inactive gene by using the methods of the present invention.

Recently, there has been advancements in nuclei transfer procedures for the generation of clonal and chimeric animals. The generation of clonal animals has been slowed because most sources of genetic material (nuclei) contain inactivated genes. Treating cells that are a source of such genetic material with methioninase, decreases the level of inactive genes, thus providing for increased efficiencies for generating clonal and chimeric animals. In such a use, the method comprises treating cells that are a source of genetic material with methioninase for a period of time sufficient to reduce methylated DNA content, prior to the use of the genetic material to generate clonal or chimeric animals.

B. Silencing Of Transgenes

It has been observed that many exogenoulsy supplied expression units that are introduced into a cell are not expressed at the level that might be expected based on the promoter and control sequences employed (Matzke, M. *et al., Plant Physiology* 107:679-685 (1995)). The inactivation of transgenes is known as silencing. One of the mechanisms that has been proposed for the silencing phenomenon is that the transgene become methylated before, or shortly after introduction, thereby inactivating the transgene. By using methioninase treatment to reduce the availability of methionine for DNA methylation reactions prior to or contemporaneously with the introduction of the transgene, the silencing phenomenon can be reduced. In such a use, a cell is preferably pretreated with methioninase prior to introduction of the transgene. Such a method reduces the level of methylation found in the introduced transgene, thus decreasing the silencing phenomenon and increasing the degree of transgene expression.

C. Increasing The Longevity Of Cells, Tissues Or Organisms

There are many situations in which it is desirable to decrease the rate at which a cell, tissue or organism differentiates, matures and ages. The following is provided as a non-limiting set of examples where such a method is applicable. A skilled artisan can readily recognize and develop other situations in which a reduction in the rate of differentiation, maturation and aging can be used.

In one use, decreasing the amount of methylated DNA that is present in an organism can be used as a means for extending the life of the organism. In such a use, the preferred organism is a mammal, preferably a human subject. By reducing the methylated DNA content in the cells of a mammal, the mammal will experience a decrease in the rate of the aging process.

In the context of human patients, long term administration of methioninase can be effective at prolonging the life of the patient.

In the context of commercially important animals, such as milk producing cows, beef cattle and chickens, methioninase can be used as a means for increasing the longevity and productivity of the animal. In such a use, methioninase is used to alter the level of methylated DNA in the organism, thus sustain milk/egg productivity.

In another use, many cell types, particularly primary cell explants, have a limited ability to be maintained in culture, displaying a high rate of cell death. The low degree of culturability of such cells is partially do to aging related mechanisms that are mediated, in part, by DNA methylation. By decreasing the methylated DNA content in cultured cells, such cells can be maintained in culture for extended periods of time. Increasing the length of time cells can be cultured is particularly important in the use of cells to produce important biomolecules.

In another use, the methods of the present invention can be used during the transport of organs and tissues used in transplant. Specifically, by including methioninase in the media used in transporting organs and tissues, the organs and tissues will have a reduced incidence of cellular apoptosis.

IV. Cell, Tissue Or Organisms With Altered Amounts Of Methylated DNA

The present invention further provides cell, tissue or organisms that have been altered using molecular techniques so that they contain and express an introduced methioninase encoding DNA expression unit which results in an altered amount of methylated DNA. As provided above, such cell, tissue or organisms will 1) express normally inactive genes, and/or 2) differentiate, develop, mature and age at a rate that is slower than non-altered cells, tissues or organisms.

The preferred cell, tissue or organisms of the present invention that have a slower rate of maturation will mature at a rate which is about 10 % to about 25% slower, more preferably about 25 % to about 50% slower, most preferably about 50 % to about 100% slower than a non-altered cell, tissue or organism.

The cell, tissue or organisms or the present invention includes those that have been altered, using molecular techniques, to have an introduced methioninase expression unit that results in an altered amount of methylated DNA sufficient to alter the rate of maturation of the cell, tissue or organism. The cell, tissue or organisms of the present invention therefore include any cell, tissue or organism that can be altered using molecular techniques so as to alter the amount of methylated DNA present in the cell, tissue or organism.

CLAIMS

1. A method to decrease the amount of methylated DNA present in a cell comprising the step of contacting said cell with a composition comprising methioninase for a time sufficient to reduce the amount of methylated DNA present in said cell when compared to non-treated cell.

2. A method to decrease the amount of methylated DNA present in a cell, tissue or organism comprising the step of genetically altering said cell, tissue or organism, using molecular techniques, so that said cell, tissue or organism expresses a methioninase encoding DNA molecule.

3. The method of claim 1, wherein said alteration results in about a 10 % to about a 25 % decrease, or greater, in the amount of methylated DNA present in said cell, tissue or organism.

4. The method of claim 2, wherein said alteration results in about a 10 % to about a 25 % decrease, or greater, in the amount of methylated DNA present in said cell, tissue or organism.

5. The method of claim 1, wherein said alteration results in about a 25 % to about a 50 % decrease, or greater, in the amount of methylated DNA present in said cell, tissue or organism.

6. The method of claim 2, wherein said alteration results in about a 25 % to about a 50 % decrease, or greater, in the amount of methylated DNA present in said cell, tissue or organism.

7. The method of claim 1, wherein said alteration results in about a 50 % or about a 70 % decrease, or greater, in the amount of methylated DNA present in said cell, tissue or organism.

8. The method of claim 2, wherein said alteration results in about a 50 % or about a 70 % decrease, or greater, in the amount of methylated DNA present in said cell, tissue or organism.

9. The method of claim 1, wherein said reduction in methylated DNA results in the activation of inactive genes.

10. The method of claim 21, wherein said reduction in methylated DNA results in the activation of inactive genes.

11. The method of claim 1, wherein said reduction in methylated DNA results in a cell that has a reduced rate of growth.

12. The method of claim 2, wherein said reduction in methylated DNA results in a cell that has a reduced rate of growth.

13. The method of claim 1, wherein said reduction in methylated DNA results in a cell that has an increase in longevity.

14. The method of claim 2, wherein said reduction in methylated DNA results in a cell that has an increase in longevity.

15. The method of claim 1, wherein said method is used with isolated cells.

16. The method of claim 2, wherein said method is used with isolated tissue.

17. A method to decrease the amount of methylated cellular constituents present in a cell comprising the step of contacting said cell with a composition

comprising methioninase for a time sufficient to reduce the amount of cellular constituents present in said cell when compared to non-treated cell.

18. A method to decrease the amount of methylated cellular constituents present in a cell, tissue or organism comprising the step of genetically altering said cell, tissue or organism, using molecular techniques, so that said cell, tissue or organism expresses a methioninase encoding DNA molecule.

19. The method of claim 17, wherein said alteration results in about a 10 % to about a 25 % decrease, or greater, in the amount of methylated cellular constituents present in said cell, tissue or organism.

20. The method of claim 18, wherein said alteration results in about a 10 % to about a 25 % decrease, or greater, in the amount of methylated cellular constituents present in said cell, tissue or organism.

21. The method of claim 17, wherein said alteration results in a decrease in the amount of methylated cellular constituents selected from the group consisting of RNA, protein, lipids and small cellular molecules.

22. The method of claim 18, wherein said alteration results in a decrease in the amount of methylated cellular constituents selected from the group consisting of RNA, protein, lipids and small cellular molecules.

23. The method of claim 17, wherein said method is used to treat obesity.

24. The method of claim 18, wherein said method is used to treat obesity.

25. The method of claim 17, wherein said method is used to treat Parkinson's disease and other neurological disorders.

26. The method of claim 18, wherein said method is used to treat Parkinson's disease and other neurological disorders.

27. The method of claim 1, wherein said method is used in generating transgenic, chimeric or clonal animals.

28. The method of claim 1, wherein said method is used in generating transgenic, chimeric or clonal animals.